NASA - TECHNICAL TRANSLATION NASA TT F-15,843

SCREENING OF ANTINUCLEAR FACTORS IN RHEUMATIC DISEASES

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Translation of "Dépistage des facteurs antinucléaires dans les maladies rhumatologiques", Union Medicale du Canada, Vol. 103, No. 4, 1974, pp. 722-726.

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON, D. C. 20546 SEPTEMBER 1974

STANDARD TITLE PAGE

1. Report No. NASA TT F-15,843	2. Government Accession No.	3. Recipient's Catalog No.
4. Title and Subtitle: SCREENING OF ANTINUCLEAR FACTORS IN RHEUMATIC DISEASES		5. Report Date: September 1974 6. Performing Organization Code
7. Author(s): H. A. Menard, D. Myhal, M. Camerlain and A. Lussier		8. Performing Organization Report No. 10. Work Unit No. 11. Contract or Grant No. NASw-2483
9. Performing Organization Name and Address: SCITRAN, Box 5456, Santa Barbara, CA 93108		13. Type of Report and Period Covered: Translation
12. Sponsoring Agency Name and Address: National Aeronautics and Space Administration, Washington, D.C. 20546		14. Sponsoring Agency Code

15. Supplementary Notes

Translation of "Dépistage des facteurs antinucléaires dans les maladies rhumatologiques", Union Medicale du Canada, Vol. 103, No. 4, 1974, pp. 722-726.

16. Abstract

The authors present their experience with a screening method for antinuclear antibodies. This method uses indirect immunofluorescence on formalinized chicken red cells nuclei as substrate. The method is inexpensive, easy to standardize, easy to perform and presents a sensitivity and specificity comparable to classical methods. Sera and synovial fluids from patients with rheumatic diseases were screened. The authors discuss the technical aspects and the clinical applications.

17. Key Words (Selected by Author(s))	18. Distribution Statement
	Unclassified - Unlimited

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price
Unclassified	Unclassified	15	

SCREENING OF ANTINUCLEAR FACTORS IN RHEUMATIC DISEASES *

H. A. Menard**, D. Myhal, M. Camerlain and A. Lussier ***

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The description of the LE cell by Hargraves in 1948 [6] has been at the basis of several methods of detection of "LE factors", or best, of antinuclear factors (FAN) over the past 25 years. The latter are immunoglobulins (Ig) of heterogeneous structures, biological properties, and antigenic specificity, represented by various components of the cell nuclei. Their presence in biological fluids is most often discovered by indirect immunofluorescence techniques [10].

The more and more frequent use of this laboratory analysis as diagnostic orientation tests has led us to develop a screening method satisfying the qualities of rapidity, simplicity, economy, specifity and sensitivity. We report here an adaptation of the method of Ten Veen and Feltkamp [11] which seems to answer those criteria. We applied it to serum and synovial fluids in a group of rheumatic patients after evaluation in normal subjects.

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MATERIAL AND METHODS

The clinical material was provided by patients in treatment at the Rheumatic Diseases Unit of the University Hospital of Sherbrooke. Clinical diagnosis was decided following the criteria of the American Rheumatic Association for the cases of disseminated lupus erythematosus (LED)* and rheumatoid polyarthritis (PR). We studied 246 patients: 148 serum samples and 98 synovial fluid samples. The FAN are detected by indirect immunofluorescence with the following technique:

Substrate preparation

Chicken red blood cells are used as substrates since these cells are nucleated. Chicken blood is collected in an equal volume of Alsevers and taken to the laboratory. After washing in Phosphate buffered saline 0.015M, pH7.2 (PBS), the red cells are treated with 0.45% saponin for 5 minutes which liberate the nuclei. Those are centrifugated down, washed and fixed in 40% formaldehyde for 48 hours. The supernatant is sucked off, and the nuclear suspension adjusted to 10% in PBS, then stored at either room temperature or 4° C. This preparation can be stored for periods of time longer than 6 months. The day before the analysis, the "stock" suspension is diluted 1:50 in PBS. A drop is placed in the center of a circle etched on a microscope slide and air dried. The slides are ready for use or can be stored for 24 hours at room temperature. After one day the autofluorescence increases progressively.

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* Meaning of abbreviations: FAN: Antinuclear factors; LED: Disseminated lupus erythematosus; PR: Rheumatoid polyarthritis; Ig: Immunoglobulin; PBS: Phosphate buffered saline; FITC: Fluorescein iso-thiocyanate.

Immunological reagents

We use a rabbit serum with anti-human Ig antibodies marked with fluorescein isothiocyanate (FITC) (Hyland Lot No: 2202 T005A1), characterized as follows: protein concentration 21.7 mg/ml, molar ratio fluorescein/protein: 4.7, titer of precipitin assay in agar against purified human Ig: 1:16. The FITC labelled goat anti-serum, with mouse anti-Ig, and the FITC goat anti-serum with rabbit anti-Ig were supplied by Hyland's. Bovine serum albumin (General Biochemical) is labelled with fluorescein by the Kawamura technique [7].

Detection test

The slides are rehydrated by PBS and a drop of the fluid to /723 test is placed on top and incubated at room temperature in a moist chamber for 20 minutes. After 3 washes of 5 minutes each in PBS, a drop of fluorescent anti-serum is added for a 20-minute incubation in the same conditions. The preparation is washed and mounted in 90% (v/v) glycerol - PBS. The observation is done on a Zeiss microscope with a fluorescence attachment, a mercury lamp HBO-200, an exciter filter KP500 and a barrier filter No. 50.

RESULTS

Standardization

The substrate suspension was adjusted to 0.2% in PBS. Non-specific background is noted on lines A, B, C of the table:

TABLE 1. GENERAL PROCEDURE AND CONTROLS

Series	1st Step	2nd Step	Results
A	PBS	FITC Reagent	-
B	Negative Serum	-id-	-
C	Positive Serum	-id-	-
D	Positive Serum	FITC Anti-human-Ig	+

If in the 1st step the nuclei are treated with PBS (A) or a negative control serum (B), none of the fluorescent compound mentioned in Material and Methods applied in the 2nd step gives a positive reaction. The same results are obtained for the FITC reagents minus anti-human Ig if a positive control serum (C) is used in the 1st step. Positive results are obtained only if a positive control serum is used in the 1st step and followed by FITC anti-serum, anti-human Ig(D). The latter diluted to 1:80 can still distinguish between a positive and a negative serum diluted 1:20. All the fluids were studied after an initial dilution of 1:20; and FITC anti-serum anti-human Ig in a standard dilution of 1:80. The samples positive at 1:20 were then titered. Each group of assays had the series A, B, and D with fluorescent serum anti-human Ig.

Interpretation of the pictures

Chicken red blood cells (Figure 1a) treated by saponin are mostly free cytoplasm and their nuclei are immediately available as antigens. It is usually easy to distinguish a negative (Figure 1b) from a positive sample (Figure 1c). If the result is doubtful, the assay is repeated with dilution of the same sample, or with another sample from the same patient.

The negative pictures (normal) show a weak greyish autofluorescence against a dark background. They are easily distinguished from the bright green (pathological) positive picture. There are two kinds of positive pictures: either an intense fluorescence, uniformly distributed over the nuclear structure, or a weakly diffuse fluorescence with sharper and brighter edges. These two types do not seem to correspond to the specificity of the detected FAN, since they both can be seen in a same sample at the same dilution, taken at different days. Furthermore, the variation bound to the dilution of the sample does not always /724 lean towards the same direction. These characteristics seem to be due to the conditions of the assay favoring more or less the diffusion of the antibody towards the antigen [12]. The serum titers obtained show a very good reproducibility from one day to another, and the total positive and negative results are always in agreement. The long storage of samples at -20° C (up to 2 years) does not change the results. However, when samples are frequently frozen and thawed out, their serum-titer decreases but rarely disappears.

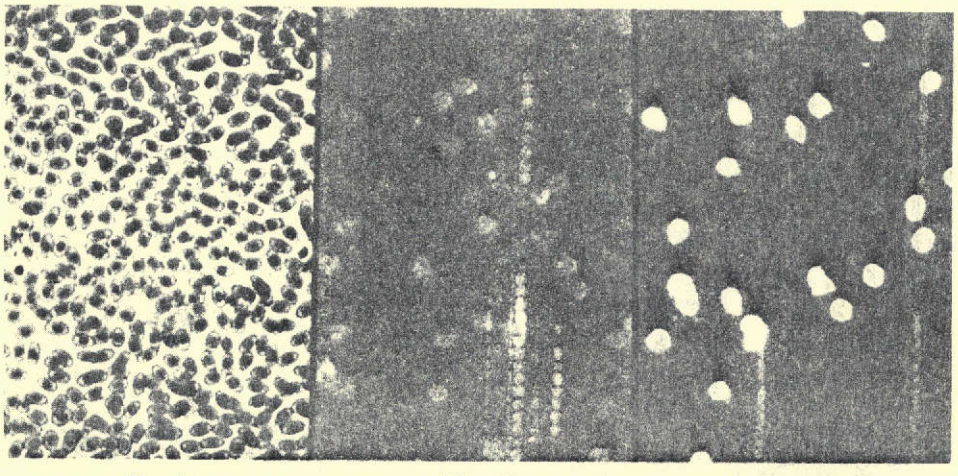

Fig. 1a Fig. 1b Fig. 1c

Figure 1. Chicken blood cells from a day smear. (a) Wright stain. Negative (b) and positive (c) serum seen with UV after reaction with FITC serum anti-human Ig (X 400).

Screening

Figure 2 shows the serologic titers in serums organized by diagnosis categories. Of the 22 controls subjects, only one (4.5%) gives a result greater than 1:20. We considered this titer as the threshold of positivity. The 11 cases of LED are all positive; among the 98 cases of PR (a juvenile negative PR), 32% are positive. The group of "miscellaneous" include 3 psoriatic arthritis, 3 osteoarthritis, 2 sarcoidosis, 2 collagenosis, 3 cases of arthralgia of unknown origin and a case each of gout, septic arthritis, bacterial endocarditis, and monoarthritis. Two only are positive: a case of mixed disease of the connective tissue, and the case of monoarthritis.

Figure 3 shows the results obtained with the synovial fluids. Here also the threshold of positivity was established as 1:20, because of the constantly negative results, at this dilution, of 18 cases of osteoarthritis. Two of the 19 cases of microcrystalline arthritis were weakly positive; in both cases the patients were alcoholics with hepatic dysfunction. In the group of PR are 7 juvenile PR, 4 among which are negative. In total, 60% are positive. Three of the 6 cases of monoarthritis are positive. The group of "miscellaneous" includes 3 ankylosed spondylarthritis, 3 psoriatic arthritis, 3 septic arthritis, 2 arthritis of unknown etiology, and a case each of Reiter syndrome and of arthritis associated with Crohn's disease. In that last group, 1 case of psoriatic arthritis, and the 2 cases of arthritis of unknown etiology are positive.

DISCUSSION

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This work serves a double purpose: to present the advantages of the technique of detection, and to report the results of screening performed within the framework of rheumatic diseases.

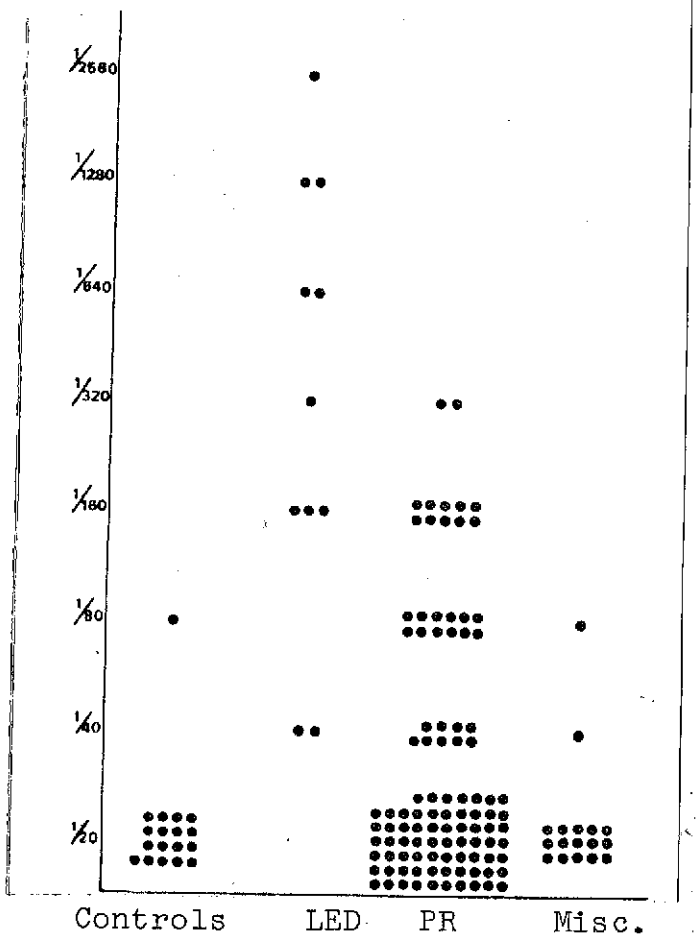

Figure 2. Distribution of the FAN serum-titers in the serum.

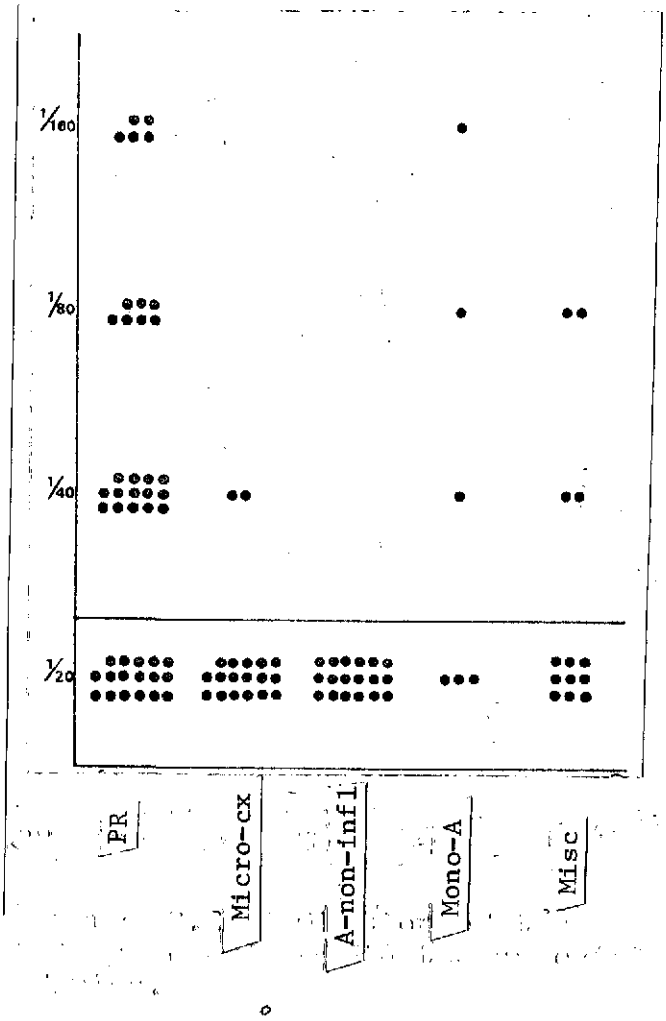

Figure 3. Distribution of the FAN serum-titers in the synovial fluids.

Technical aspects

The classical technique is performed on animal organ, quick frozen in liquid nitrogen or dry ice, and stored at low temperature (-20° to -70° C) for an undetermined period of time, but in a cryostat and mounted on a slide. This technique requires the following facilities: animal quarters, a cold storage tank, material for quick freezing, a cryostat, and finally a qualified and skilled person to cut the sections. Our technique requires absolutely free material (the chicken slaughter-houses discard the blood), which can be prepared in four days only, and then be stored 6 months at room temperature. The slides are ready in a few minutes and necessitate no special skill. The method is very sensitive and by then inexpensive since the fluorescent reagent is used at a 1:80 dilution. A last sophistication to bring would be to replace completely the fluorescent by an enzymatic marker. Preliminary experiments with peroxidase labelled antibodies allow us to think of this possibility. But the results obtained are scattered and show a great loss of sensitivity. One of the reasons is the weak ratio of peroxidase labelled antibodies in each anti-serum [8] so that only undiluted must be used; and only the serum positive for 1:160 by immunofluorescence are positive. We think, however, that this technique could be feasible if the reagents could be improved.

A possible disadvantage of the technique is that with this substrate, no "pattern" or mode of distribution of fluorescence can be observed. The notion of "patterns" introduced by Beck implies the FAN specificity for a given antigenic structure [3]. These notions, in their whole, have a definite statistical value; however, the modes of fluorescence distribution do not always correspond to the antigenic specificity that they suggest. Furthermore, most of the time, the patient's serum contains simultaneously several types of FAN, though the most important

quantitatively only will show up at the assay. None of the "patterns" correspond absolutely to a given nosologic framework. In view of these considerations, of the difficulty of standardization, and of the technical skill that the interpretation of those "patterns" implies, we have sacrificed this last information.

Our attitude at this point is to use this method, more economical, technically easier and faster, to screen the FAN (test of sensitivity). If the clinical data suggest it, we perform a test of specificity to look for, for instance, anti-DNA antibodies (LED) [11] or anti-ENA antibodies (mixed disease of the connective tissue) [12].

Clinical applications

It has become evident during the last few years, that the presence of FAN in a serum does not have a pathognomonic value. Its only clinical value is for the orientation of the diagnosis. We then had to develop a screening assay which results could be interpreted in the light of the clinical findings. In the case of LED for instance, the common opinion is that this diagnosis is doubtful when serum FAN cannot be found; and on the opposite, a positive FAN is not pathognomonic of LED [2]. Several systemic diseases called "septic systemic angiitis" present similar symptoms to LED without being in the same nosologic framework. If we compare our results to those reported by classical methods, we see that all our cases of LED are positive, and that in more recent and important series, the incidence is 99.5%.

On the opposite, for PR, the precise incidence of FAN in serum and synovial fluid is more difficult to appreciate, and seems to vary with the nuclear substrate. If the substrate consists of a cryostat section, it is agreed that about 30% of the cases will be positive in the serum and the synovial fluid [4,5,9,13]. If the substrate consists of peripheral blood leukocytes, the incidence, in both serum and synovial fluid, is doubled, i.e., about 50-65% [1, 4, 15]. Our results indicate 32% in serum and 59.5% in synovial fluid. Two comments are necessary to clarify those differences. First of all, it seems that in PR, the FAN have a specificity for granulocytes [4] and we probably would not detect those with the chicken red cells nuclei. The incidence in the rheumatoid serum is then 30% closer with our technique. Second, our results of 60% positive in the synovial fluid is explained, partly, because those assays are performed on frozen-thawed fluids, without separation of the cellular elements present. This is compatible with the results of Zvaifler [14] who finds FAN in the lysates of rheumatoid synovial fluid leukocytes which were judged negative as a whole. Practically, if the weaker titers are neglected to keep only the titers greater than 1:40, the incidence of FAN in rheumatoid synovial fluid is about 37%. On the other hand, almost 90% of the fluids positive at 1:80 or more belong to the group of PR and monoarthritis where long-term outcome is presently unknown.

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The clinical interest in the determination of synovial fluid FAN is not established by these datas. But the following example encourages us to pursue their evaluation. The case was the one of a young woman treated in another hospital for a knee hydrarthrosis. The medical history, clinical x-rays, datas, pointed to a mechanical problem, and a menistectomy was performed. In fact, the surgery consisted in synovectomy of a succulent membrane that histopathology showed to be filled with lympho-plasmo histiocytes. The synovial fluid FAN were 1:60 in the preoperatory period. In

retrospect, the same patient had been in a third hospital for a medico resistant oleocranian bursitis and surgery was performed. The pathologist had mentioned a diagnostic of PR in front of similar findings. Since then, we started a special study of the monoarthritis in order to evaluate the nosologic and prognostic value of the FAN. These results indicate that a possible physio-pathological role can be defined for the FAN, at the local level, in the inflamatory joint affections.

SUMMARY

The authors present their experience with a screening method for antinuclear antibodies. The method uses indirect immuno-fluorescence on formalinized chicken red cells nuclei as substrate. The method is inexpensive, easy to standardize, easy to perform and has a sensitivity and specificity comparable to classical methods. Sera and synovial fluids from patients with rheumatic diseases were screened. The authors discuss the technical aspects and the clinical applications.

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Translated for National Aeronautics and Space Administration under contract No. NASw 2483, by SCITRAN, P. O. Box 5456, Santa Barbara, California, 93108.